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Original article

Synthesis, physicochemical properties and biological evaluation of ester prodrugs of 3-hydroxypyridin-4-ones: design of orally active chelators with clinical potential

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Abstract – The synthesis of a range of hydrophobic ester prodrugs of 3-hydroxypyridin-4-ones with potential for oral administration is described. The distribution coefficient values of a range of these ester prodrugs and the corresponding alcohols in 1-octanol and MOPS buffer pH 7.4 are presented together with their rates of hydrolysis at pH 2, pH 7.4, in rat blood and liver homogenate. In vivo iron mobilisation efficacy of the pivaloyl and benzoyl prodrugs has been compared with their parent drugs using a ^{59}Fe-ferritin loaded rat model. Both classes of prodrug enhanced the ability of the parent hydroxypyridinone to facilitate the excretion of ^{59}Fe. The influence of the pivaloyl function was more marked than that of the benzoyl function. The optimal effect was observed with 1-[2´-(pivaloyloxy)ethyl]-2-methyl-3-hydroxy-4(1*H*)-pyridinone **25**. However, not all the prodrugs provide increased efficacy which suggests that lipophilicity is not the only factor which influences the drug efficacy. The metabolism of the compound may have a dominating influence on the overall efficacy. © Elsevier, Paris

hydroxypyridinone / prodrug / iron chelators

1. Introduction

The preferred treatment of β-thalassaemia major is to maintain high levels of haemoglobin by regular blood transfusion [1]. Repeated transfusion unfortunately leads to elevated body iron levels due to the inability of humans to excrete iron via the kidney [2]. Excess iron is mainly located within the liver and other highly perfused organs and this leads to tissue damage, organ failure and eventually death [3]. Complications associated with elevated iron levels can be largely avoided by the use of iron-specific chelating agents and in particular desferrioxamine (DFO) [4, 5]. The major limiting factor of DFO is that it is not orally active and has to be administered parentally [6]. This in turn leads to poor patient compliance.

3-Hydroxypyridin-4-ones (HPOs) are selective for iron (III) under most biological conditions, but unlike DFO, are efficiently absorbed, when administered orally [7, 8]. The simple 1,2-dialkyl derivatives, such as CP20 (Deferiprone, L1) (**1**), are highly effective at removing iron from iron overloaded animals [9] including man [10, 11] but are associated with two disadvantages; (i) they penetrate cells easily and therefore gain ready access to the bone marrow and the brain [12] and (ii) they are rapidly conjugated with glucuronic acid, thereby loosing their iron binding properties [13]. Penetration of membranes can be effectively decreased by designing more hydrophilic hydroxypyridinones, for instance hydroxyalkyl hydroxypyridinones [14]. Some of 1-hydroxyalkyl derivatives of HPOs, such as CP102 (**2**), CP40 (**3**) and CP41 (**4**) *(table I)*, are not rapidly metabolised by Phase II mechanisms and therefore their chelating action is more prolonged [15]. Not surprisingly the increased hydrophilicity of such compounds is associated with a decreased efficiency of absorption from the gastrointestinal tract and more seriously, reduced liver extraction. Efficient liver extraction of a chelator leads to

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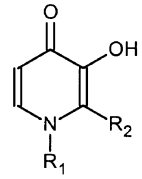
Abbreviations: DFO, desferrioxamine; HPO, hydroxypyridinone; DEAD, diethyl azodicarboxylate; $D_{7.4}$, distribution coefficient at pH 7.4; GIT, gastrointestinal tract; HPV, hepatic portal vein; MOPS, 4-morpholinepropane sulphonic acid; PBS, phosphate buffer; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SD, standard deviation.

Table I. Chemical structure of CP20 (Deferiprone) and several 1-hydroxyalkyl hydroxypyridinones.



Compound	R1	R2	$D_{7.4}$
CP20 (**1**)	CH_3	CH_3	0.17
CP102 (**2**)	CH_2CH_2OH	CH_2CH_3	0.22
CP40 (**3**)	CH_2CH_2OH	CH_3	0.08
CP41 (**4**)	$(CH_2)_3OH$	CH_3	0.13

high biliary iron-excretion rates, a highly desirable feature for the therapy of iron overload [16].

This difficulty can, in principle, be overcome by the use of prodrugs, whereby a hydrophobic prodrug is absorbed from the gastrointestinal tract and then efficiently extracted by the liver during the "first pass". Once in the hepatocyte, the ester link is cleaved to a much more hydrophilic chelator *(figure 1)*. Preliminary studies with hydrophobic ester prodrugs of 1-hydroxyalkyl hydroxypyridinones have demonstrated that they possess suitable pharmacokinetics for further consideration [17]. In this work we describe the synthesis, characterisation and efficacy of a range of such hydrophobic ester prodrugs of 1-hydroxyalkyl hydroxypyridinones.

2. Chemistry

The 1-(hydroxyalkyl)-2-alkyl-3-benzyloxy-4(1*H*)-pyridinones **9–11,** were prepared by following the methodology as described by Rai and co-workers [14] *(figure 2)*. The commercially available maltol (**5**) or ethyl maltol (**6**) were benzylated in 90% aqueous methanol to give **7** and **8**. Refluxing **7** or **8** with ethanolamine or 3-hydroxylpropylamine in aqueous ethanol, in the presence of a catalytic amount of sodium hydroxide, gave the benzylated alcohols **9–11** which were isolated as the free-bases.

The synthesis of HPO esters, 1-alkanoyloxy- and 1-benzoyloxyalkyl-2-alkyl-3-hydroxy-4(1*H*)-pyridinones was accomplished using two methods, either from acid chlorides *(figure 3)* or carboxylic acids via an alkyltriphenylphosphonium carboxylate intermediate *(figure 4)*. The reaction of benzyl protected alcohols with acid chlorides in the presence of catalytic bases, such as triethylamine [18] or pyridine [19], afforded diesters

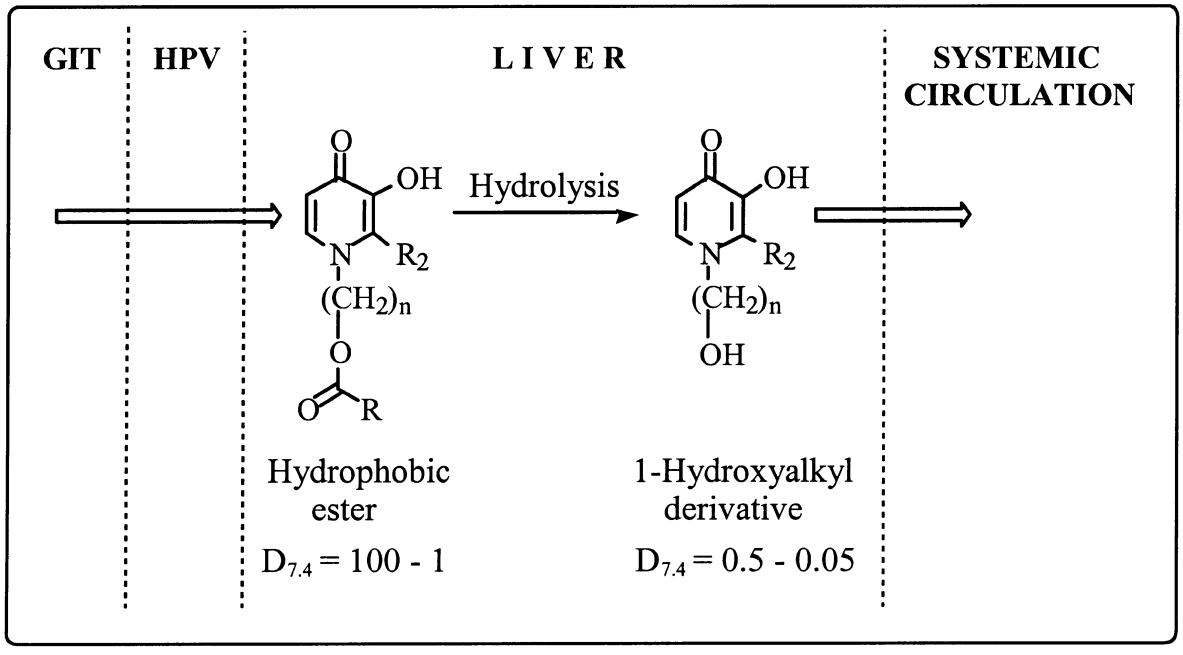


Figure 1. Schematic representation of the use of ester prodrugs of 1-hydroxyalkyl HPOs to enhance both the absorption from the gastrointestinal tract (GIT) and the liver extraction from the hepatic portal vein (HPV) of the prodrug. Subsequent intracellular hydrolysis occurs yielding a hydrophilic chelator.

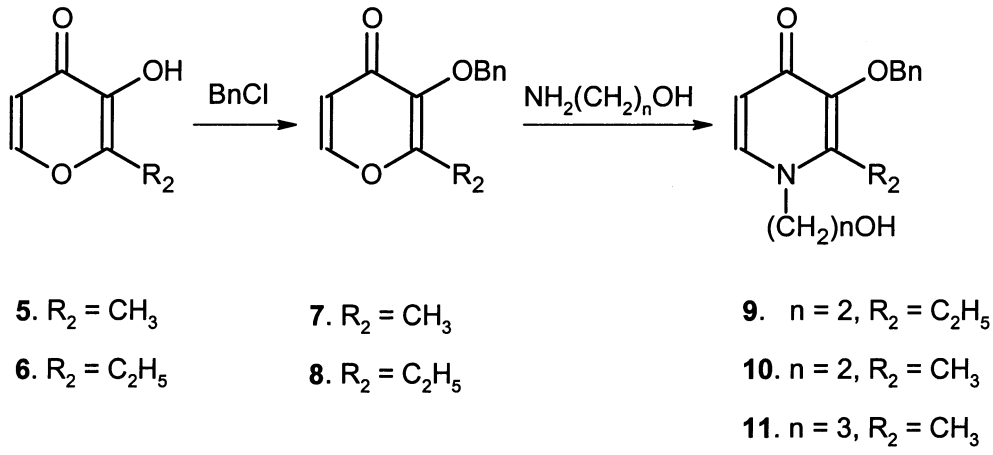


Figure 2. Synthetic methodology adopted for the preparation of the benzyl protected alcohols, 1-(hydroxyalkyl)-2-alkyl-3-benzyloxy-4(1*H*)-pyridinones **9–11**.

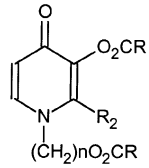
12–**15** *(table II)* at elevated temperatures and not the expected benzyl protected esters, as a result of cleavage of the benzylic carbon-oxygen bond. Hydrolysis of diesters in water generated the desired ester chelators *(table III)*. At room temperature a mixture of benzyl protected ester and diester was formed *(figure 3)*. Examples of ether cleavage by pyridine hydrochloride have been previously reported [20, 21]. Triethylamine hydrochloride probably plays a similar role to that of pyridine hydrochloride. In contrast, the direct use of the benzyl protected alcohol and carboxylic acids in the presence of diethyl azodicarboxylate (DEAD)/triphenylphosphine led to the formation of the desired benzyl protected esters **16–20** in good yields *(figure 4)*. This reaction is believed to proceed through the formation of a quaternary phosphonium salt, by addition of triphenylphosphine to DEAD, which reacts in situ with alcohols in the presence of a carboxylic acid to form an alkoxytriphenylphosphonium carboxylate. The phosphonium moiety is then displaced by the carboxylate anion to give the desired ester product [22, 23]. Removal of the benzyl group was achieved by catalytic hydrogenation to yield the bidentate chelators, which were isolated as free bases or hydrochloride salts *(table III)*.

3. Physicochemical properties and biological experiments

3.1. Determination of solution properties

Since one of the critical parameters of ester prodrugs is the lipophilicity of the compounds, the determination of distribution coefficients (D values) of prodrugs is important. Distribution coefficients of the chelators were measured using a buffered octanol/aqueous system. A modified automated continuous flow technique [7, 24] was

Table II. Synthesis of 1-alkanoyloxyalkyl-2-alkyl-3-alkanoyloxy-4(1H)-pyridinones in the presence of triethylamine[1] or pyridine[2] as catalytic bases.



Compound	R_2	R	*n*	m.p. (°C)	Yield (%)	Formula
12	C_2H_5	$C(CH_3)_3$	2	141–142	79[1]	$C_{19}H_{29}NO_5$
13	C_2H_5	$CH(CH_3)_2$	2	133.5–134.5	77[1]	$C_{17}H_{25}NO_5$
14	CH_3	$C(CH_3)_3$	2	163–164.5	74[2]	$C_{18}H_{27}NO_5$
15	CH_3	$C(CH_3)_3$	3	169–170	71[2]	$C_{19}H_{29}NO_5$

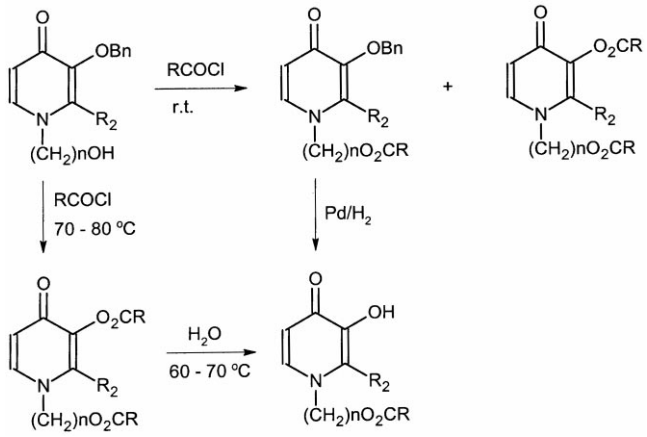


Figure 3. Synthesis of ester prodrugs via acid chlorides, method (a).

chosen in preference to the traditional shake flask method owing to a greater accuracy and reproducibility of measurements.

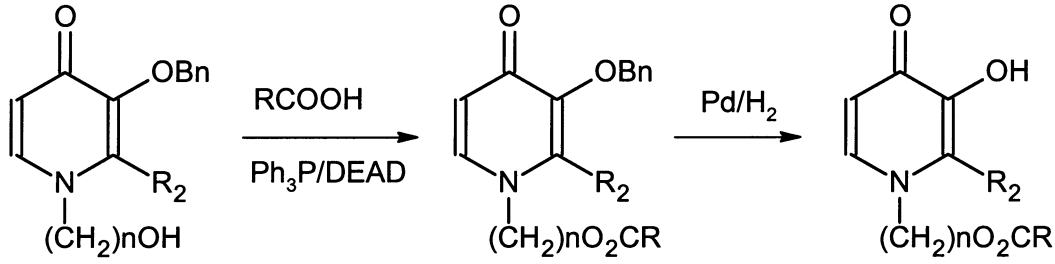
3.2. Biological experiments

3.2.1. Stability study

In order to obtain selective delivery of the chelator to the major chelatable iron pool in the body (the liver), it is essential that prodrug esters are absorbed intact from the gastrointestinal tract, and are reasonably stable in the plasma, thereby providing sufficient time for the prodrug to perfuse the liver. Subsequently, rapid hydrolysis by hepatic carboxyesterases will generate hydrophilic metabolites within hepatocytes. In order to identify a lead prodrug by which specific drug delivery can be achieved, it is essential to determine the stability of this ester in different conditions. Preliminary in vitro esterase studies of the aliphatic ester derivatives of CP102 (**2**), namely **21**, **22** and **23**, indicate that the pivaloyl ester analogue **23** may partially fulfil the requirements for relatively efficient liver extraction. This is due to its resistance towards hydrolysis in pH 2 and 7.4 buffer and a much lower hydrolysis rate in plasma than the liver, whereas **21** and **22** show much faster hydrolysis rates in plasma [25]. In this current study, the hydrolysis rates of pivaloyl ester derivatives **23**, **25** and **27** were compared with their benzoyl analogues **24**, **26** and **28** at different pHs (pH 2 and pH 7.4), in rat blood and liver homogenate.

3.2.2. Efficacy study

In vivo iron mobilisation efficacy of all HPO ligands has been measured in a non-iron overloaded rat model. ^{59}Fe-ferritin has been used to label the liver iron pool [26], followed by a challenge with test chelator at a time when iron released by lysosomal degradation of ferritin is maximally available. Since the major reason to



16. n = 2, $R_2 = C_2H_5$, $R = CH_3$

17. n = 2, $R_2 = C_2H_5$, $R = C(CH_3)_3$

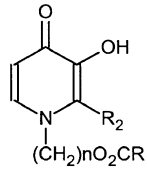
18. n = 2, $R_2 = C_2H_5$, $R = C_6H_5$

19. n = 2, $R_2 = CH_3$, $R = C_6H_5$

20. n = 3, $R_2 = CH_3$, $R = C_6H_5$

Figure 4. Synthesis of ester prodrug via carboxylic acid, method (b).

Table III. Synthesis of 1-alkanoyloxy- and 1-benzoyloxyalkyl-2-alkyl-3-hydroxy-4(1*H*)-pyridinones and their distribution coefficients between an aqueous phase buffered at pH 7.4 and octanol.



Compound	R2	R	*n*	m.p. (°C)	Yield (%)	Formula	$D_{7.4}$
21	C_2H_5	CH_3	2	126–127	88[1]	$C_{11}H_{15}NO_4$	0.58 ± 0.01 (*n* = 6)
22	C_2H_5	$CH(CH_3)_2$	2	106–107	96[2]	$C_{13}H_{19}NO_4$	5.06 ± 0.09 (*n* = 6)
23	C_2H_5	$C(CH_3)_3$	2	133–134	90[1]; 92[2]	$C_{14}H_{21}NO_4$	14.5 ± 0.08 (*n* = 6)
24	C_2H_5	C_6H_5	2	173–174	92[1]	$C_{16}H_{17}NO_4$	32.8 ± 1.68 (*n* = 6)
25	CH_3	$C(CH_3)_3$	2	139–140	92[2]	$C_{13}H_{19}NO_4$	4.70 ± 0.04 (*n* = 6)
26	CH_3	C_6H_5	2	201–203	92[1]	$C_{15}H_{16}NO_4Cl$	10.9 ± 1.05 (*n* = 6)
27	CH_3	$C(CH_3)_3$	3	156–157	80[2]	$C_{14}H_{21}NO_4$	8.78 ± 0.23 (*n* = 6)
28	CH_3	C_6H_5	3	168–170	94[1]	$C_{16}H_{18}NO_4Cl$	17.6 ± 1.31 (*n* = 6)

[1]Yield obtained from hydrogenolysis reaction of the benzyl protected ester, [2]Yield obtained from hydrolysis of the diester.

develop ester prodrugs of HPOs is to target the liver iron pool, the ^{59}Fe-ferritin/rat model is considered to be a suitable system for evaluation of the iron mobilisation efficacy of such models.

4. Results

4.1. Distribution coefficients

Values of distribution coefficients of the ester prodrugs between an aqueous phase buffered at pH 7.4 and octanol are presented in *table III*. The prodrugs are more lipophilic than the alcohols, as expected. In general, the expected increase in distribution coefficient was observed with the increase in the bulk of the ester function. Clearly, the pivaloyl esters and benzoate esters are more lipophilic and as a result are predicted to be efficiently absorbed from the gastrointestinal tract and extracted by the liver.

4.2. Stability study

The preliminary hydrolysis studies of ester prodrugs at different pH values in rat blood and rat liver homogenate are presented in *table IV*. All six ester prodrugs possess appreciable stability at both pH 7.4 and pH 2. The hydrolysis rates of these ester derivatives by rat plasma are more rapid than in PBS, falling in the range 185.6–783.5 nmoles/mL/h. The pivaloyl ester **23** was the most stable ester, with a plasma esterase activity of 185.6 nmoles/mL/h. The rate of hydrolysis of all esters was found to be much greater in the liver and a wide variety of hydrolytic rates were shown, ranging from 27 600 nmoles/g tissue/h (**23**) to 481 000 nmoles/g tissue/h (**24**). The hydrolytic rates of benzoyl ester derivatives in the liver are much faster than their pivaloyl ester analogues. Generally the hydrolytic rates of these esters in the liver were several thousand fold faster than that in the rat blood. This rapid hydrolysis in the liver will generate the parent hydrophilic alcohols.

4.3. Efficacy study

The iron mobilisation efficacy of pivaloyl (**23**, **25** and **27**) and benzoyl ester prodrugs (**24**, **26** and **28**) were compared with their parent drugs **2**, **3** and **4**, and the 1,2-dimethyl derivative **1** *(figure 5)*. Clearly, all the ester prodrugs were found to be superior to **1**. Many prodrugs provide a clear improvement over their parent compounds in the efficacy model. The pivaloyl ester **23** was found to increase efficacy from 16.7 to 23%; **25** increased efficacy from 7.6 to 35.9% and **27** increased efficacy from 29.9 to 39.3%. An enhanced iron excretion was also produced with the benzoyl ester prodrugs, for instance, **26** provides 16.9% iron excretion, compared with 7.6% for **3,** and **28** increased efficacy from 29.9 to 36.4%. Such improvement indicates that selective delivery of the drug to the liver has been achieved. However, not all the prodrugs provide increased efficacy, thus **24** is less effective than the parent compound **2**, suggesting that lipophilicity is not the only factor which influences the drug efficacy.

Table IV. Hydrolysis of ester prodrugs at different pHs, in rat blood and rat homogenate. Values are expressed as means ± SD (n = 3).

Compound	Rate of hydrolysis			
	pH 2.0 (nmole/ml/h)	pH 7.4 (nmole/ml/h)	Rat blood (nmole/ml/h)	Rat liver (nmole/g/h)
23	5.0 ± 1.1	0	185.6 ± 27.2	27 600 ± 700
24	1.9 ± 0.5	0.8 ± 0.3	591.2 ± 16.0	481 000 ± 15 200
25	43.7 ± 0.8	3.7 ± 0.2	285.9 ± 58.9	64 800 ± 2 500
26	0.7 ± 0.4	0.5 ± 0.2	783.5 ± 115.8	325 170 ± 6 300
27	12.0 ± 4.2	23.3 ± 3.0	479.2 ± 24	91 300 ± 5 200
28	0.6 ± 0.5	0.3 ± 0.1	364.0 ± 14.4	230 131 ± 2 363

5. Discussion

The esters investigated for stability in aqueous solution at pH values 2.0 and 7.4 were found to be relatively stable *(table IV)*. This is in marked contrast to esters of the 3-hydroxyl function of the pyridinones, which are unstable [27]. Thus, esters positioned at the *N*-alkyl function appear to be suitable for formulation purposes and are predicted not to be unduly unstable in the lumen of the stomach. The stability of the esters in blood was found to be sufficient to permit delivery to the liver via the portal stream, the most stable ester being the pivaloyl derivative **23** *(table IV)*. In the liver homogenate, all esters underwent rapid hydrolysis yielding the more hydrophilic hydroxypyridinone. Thus in principle the esters described in this work might be expected to be readily absorbed from the gastrointestinal tract by virtue of their favourable log D values (0–1.5) and be extracted into the hepatocyte, without undergoing appreciable hydrolysis in the lumen of the stomach or the blood perfusing the liver.

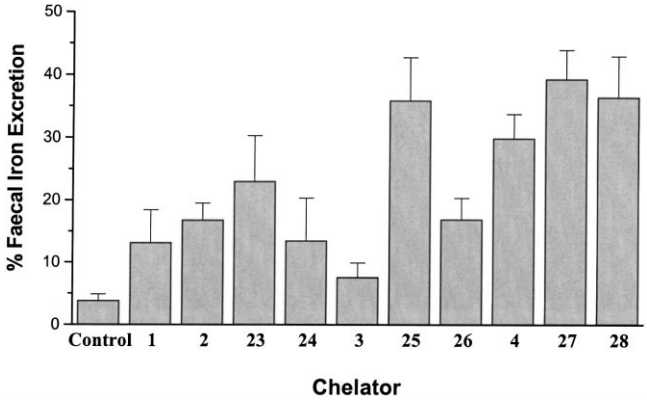


Figure 5. Iron mobilisation comparison studies between ester prodrugs and the parent 1-hydroxyalkyl HPOs in the ^{59}Fe-ferritin loaded rat model. All chelators were given orally; the chelator doses were 450 μmol/kg. Values are expressed as means ± SD (n = 5–9).

Three series of pyridinones were investigated in the ^{59}Fe-ferritin model, the hydroxyalkyl derivatised HPOs, **2**, **3** and **4** together with their pivaloyl esters, **23**, **25** and **27** and benzoyl esters, **24**, **26** and **28**. Preliminary analysis of the results shows that in each case the pivaloyl ester leads to superior iron excretion via the bile than does the corresponding benzoyl ester *(figure 5)*. This effect is particularly marked with the 1-[2´-(hydroxy)ethyl] derivative **3** where the iron excretion is increased from 7.6% to 35.9%. This enhancement is almost certainly due to the increased D_{value} of pivaloyl ester **25**, 4.7 as compared to 0.08 for the parent HPO (**3**). The reason for the more marginal increase with the benzoyl ester **26** is not clear. The D_{value} of **26** is higher than that of the pivaloyl ester **25** and therefore absorption from the gastrointestinal tract might be anticipated to be enhanced. However the benzoyl esters possess lower aqueous solubilities than the pivaloyl esters and this might adversely influence absorption; furthermore, based on the stability studies with **24** and **26**, the benzoyl esters are found to be hydrolysed more rapidly in the blood.

Although most ester prodrugs provide a clear improvement over the parent hydroxyalkyl compound, some prodrugs only induce a marginal or even negative effect. This may result from the different metabolism between ester prodrug and the parent compound. Although 1-hydroxyalkyl HPOs such as CP102 (**2**) and CP41 (**4**) do not undergo glucuronic conjugation [15], their ester derivatives, which possess different physico-chemical properties, may be involved in different metabolic pathways. Normally, esters are predicted to be hydrolysed rapidly in the liver. However, with some of the ester prodrugs, there may exist a competition between ester hydrolysis and phase II metabolism. Metabolic studies need to be undertaken in order to verify this hypothesis.

In summary, the iron mobilisation efficacy can be improved by the introduction of a prodrug strategy, but the selection of ester moieties is critical. The metabolism of the compound may have a dominating influence on the

overall efficacy. Detailed metabolic studies need to be undertaken in order to identify a lead prodrug for further development.

6. Experimental protocols

6.1. Chemistry

Melting points are uncorrected. IR spectra are recorded on a Perkin Elmer 298. Proton NMR spectra were determined with Perkin-Elmer R32 (90 MHz). Mass spectra (EI) were recorded on a Joel AX505W. Elemental analyses were performed by Butterworth Laboratories Limited, Teddington, Middlesex or Micro analytical laboratories, Department of Chemistry, The University of Manchester, Manchester, M13 9PL.

6.1.1. General procedure for the synthesis of the 1-(hydroxyalkyl)-2-alkyl-3-benzyloxy-4(1H)-pyridinones

The benzyl protected alcohols **9–11**, 1-(hydroxyalkyl)-2-alkyl-3-benzyloxy-4(1*H*)-pyridinones *(figure 1)* were prepared by following the methodology as described by Rai and co-workers [14] from commercially available maltol (**5**) or ethyl maltol (**6**).

6.1.2. Synthesis of ester derivatives of 1-(hydroxyalkyl)-2-alkyl-3-benzyloxy-4(1H)-pyridinones. Method (a) (via acid chlorides)

6.1.2.1. 1-[2′-(Pivaloyloxy)ethyl]-2-ethyl-3-pivaloyloxy-4(1H)-pyridinone **12**

Triethylamine (8.6 g, 85 mmol) was added to a solution of **9**, 1-[2′-(hydroxy)ethyl]-2-ethyl-3-benzyloxy-4(1*H*)-pyridinone free base (4.65 g, 17 mmol) in dimethylformamide (100 mL) under nitrogen atmosphere at room temperature. Pivaloyl chloride (trimethylacetyl chloride, 8.2 g, 68 mmol) was added dropwise and the mixture was heated at 75–78 °C for 22 h. The reaction mixture was cooled to 20 °C, triethylamine hydrochloride was removed by filtration and dimethylformamide was removed by rotary evaporation under high vacuum to give a reddish brown oil. The product was taken into dichloromethane (200 mL), washed with aqueous sodium bicarbonate (5% w/v; 3 × 100 mL) and water (2 × 100 mL). The organic fraction was dried over anhydrous sodium sulphate, filtered and concentrated to dryness under reduced pressure to give a reddish brown oil which solidified on standing at room temperature. Recrystallisation from ethyl acetate after treatment with decolourising charcoal yielded colourless plates (4.7 g, 79%); m.p. 141–142 °C; IR (Nujol): 1 735 (ester C=O), 1 720 (ester C=O), 1 620 (pyridinone C=O), 1 580 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.17 [9H, s, -C**(CH_3)$_3$**], 1.19 (3H, t, CH_2**CH_3**, *J* = 6.0Hz), 1.38 [9H, s, -C**(CH_3)$_3$**], 2.62 (2H, q, **CH_2**CH_3, *J* = 6.0Hz), 4.0–4.4 (4H, m, N**CH_2CH_2**O), 6.34 (1H, d, **5-H**, *J* = 8.0Hz), 7.34 (1H, d, **6-H**, *J* = 8.0Hz); MS (EI): m/z, 352 [$(M + H)^{+\cdot}$]; Anal. Calcd. for $C_{19}H_{29}NO_5$: C, 64.93; H, 8.31; N, 3.98 Found C, 65.24; H, 8.12; N, 3.98%.

Analogous procedure using **9** and isobutyryl chloride yielded **13** in a yield of 77% *(table II)*.

6.1.2.2. 1-[2′-(Isobutyryloxy)ethyl]-2-ethyl-3-(isobutyryloxy)-4(1H)-pyridinone **13**

M.p. 133.5–134.5 °C; IR (Nujol): 1 735 (ester C=O), 1 625 (pyridinone C=O), 1 580 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.13 [6H, d, -CH(**CH_3)$_2$**], 1.18 (3H, t, CH_2**CH_3**, *J* = 6.0Hz) 1.33 [6H, d, -CH(**CH_3)$_2$**], 2.2–3.1 (3H, m, **-CH**$(CH_3)_2$ & **CH_2**CH_3), 4.0–4.4 (4H, m, N**CH_2CH_2**O), 6.35 (1H, d, **5–H**, *J* = 8.0Hz), 7.32 (1H, d, **6–H**, *J* = 8.0Hz); MS (EI): m/z, 323 [$M^{+\cdot}$]; Anal. Calcd. for $C_{17}H_{25}NO_5$: C, 63.13; H, 7.79; N, 4.33 Found C, 63.54; H, 8.07; N, 4.41%.

6.1.2.3. 1-[2′-(Pivaloyloxy)ethyl]-2-methyl-3-pivaloyloxy-4(1H)-pyridinone **14**

The alcohol **10**, 1-[2′-(hydroxy)ethyl]-2-methyl-3-benzyloxy-4(1*H*)-pyridinone, free base (1.85 g, 7 mmol) was added to a solution of dimethylformamide (100 mL) containing pyridine (5.6 g, 70 mmol, 10 eq) and cooled to 0 °C under a nitrogen atmosphere. Pivaloyl chloride (3.8 g, 28 mmol, 4 eq) dissolved in dimethylformamide (5 mL) was added dropwise via a syringe with stirring, the reaction mixture was allowed to warm slowly to 20 °C and then heated at 60 °C for 12 h. Pyridine was removed azeotropically with benzene and dimethylformamide was removed under high vacuum. The dark green residue was dissolved in dichloromethane (150 mL) and washed with aqueous sodium bicarbonate (5% w/v, 4 × 100 mL) and water (2 × 100 mL). The organic fraction was dried over anhydrous sodium sulphate, filtered and the solvent was removed in vacuo to give a brown oil which solidified on standing at room temperature. Recrystallisation from ethyl acetate/decolourising charcoal afforded colourless crystals (1.75 g, 74%); m.p. 163–164.5 °C; IR (Nujol): 1 745 (ester C=O), 1 635 (pyridinone C=O), 1 590 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.18 [9H, s, -C**(CH_3)$_3$**], 1.40 [9H, s, -C**(CH_3)$_3$**], 2.25 (3H, s, **2-CH_3**), 4.0–4.4 (4H, m, N**CH_2CH_2**O), 6.35 (1H, d, **5-H**, *J* = 7.5Hz), 7.30 (1H, d, **6-H**, *J* = 7.5Hz); MS (EI): m/z, 337 [$M^{+\cdot}$]; Anal. Calcd. for $C_{18}H_{27}NO_5$: C, 64.07; H, 8.06; N, 4.15 Found C, 64.29; H, 8.33; N, 4.02%.

Analogous reaction using **11** with pivaloyl chloride afforded **15** in 71% yield *(table II)*.

6.1.2.4. 1-[3´-(Pivaloyloxy)propyl]-2-methyl-3-(pivaloyloxy)-4(1H)-pyridinone **15**

M.p. 169–170 °C; IR (Nujol): 1 745 ester (C=O), 1 724 (ester C=O), 1 630 (pyridinone C=O), 1 580 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.22 [9H, s, -C**$(CH_3)_3$**], 1.39 [9H, s, -C**$(CH_3)_3$**], 1.85–2.18 (2H, m, NCH_2**CH_2**CH_2O), 2.23 (3H, s, **2-CH_3**), 3.93 (2H, t, N**CH_2**CH_2CH_2O), 4.14 (2H, s, N CH_2CH_2**CH_2**O), 6.36 (1H, d, **5-H**, *J* = 7.5Hz), 7.25 (1H, d, **6-H**, *J* = 7.5Hz); MS (EI): m/z, 351 [$M^{+\cdot}$]; Anal. Calcd. for $C_{19}H_{29}NO_5$: C, 64.93; H, 8.31; N, 3.98 Found C, 65.07; H, 8.11; N, 3.79%.

6.1.2.5. 1-[2´-(Pivaloyloxy)ethyl]-2-ethyl-3-hydroxy-4(1H)-pyridinone **23**

The pivaloyloxy diester **12** (4.2 g, 12 mmol) was heated in water (300 mL) at 65–70 °C for 4 h. Water was removed by rotary evaporation to yield a solid residue. Recrystallisation from ethyl acetate after treatment with decolourising charcoal afforded colourless plates (2.95 g, 92%); m.p. 133–134 °C; IR (Nujol): 3 130 (OH), 1 720 (ester C=O), 1 580 (C=C) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.18 [9H, s, -C**$(CH_3)_3$**], 1.27 (3H, t, CH_2**CH_3**, *J* = 6.0Hz), 2.85 (2H, q, **CH_2**CH_3, *J* = 6.0Hz), 4.02–4.45 (4H, m, N**CH_2CH_2**O), 5.25 (1H, br, **OH**), 6.37 (1H, d, **5-H**, *J* = 8.0Hz), 7.25 (1H, d, **6-H**, *J* = 8.0Hz); MS (EI): m/z, 268 [$(M + H)^{+\cdot}$]; Anal.Calcd. for $C_{14}H_{21}NO_4$: C, 62.89; H, 7.91; N, 5.23 Found C, 62.72; H, 7.66; N, 5.38%.

Analogous procedure using **13**, **14** and **15** yielded **22, 25** and **27** respectively *(table III)*. The hydrochloride salts were prepared by dissolving the corresponding free bases in dry chloroform or ethyl acetate or dimethylformamide. Hydrogen chloride gas was passed until the lightly coloured solution became colourless. Dry diethylether was added dropwise until a faint opalescence appeared, and cooled at 0 °C for 4 h. The corresponding hydrochloride salts were then separated by filtration and dried.

6.1.2.6. 1-[2´-(Isobutyryloxy)ethyl]-2-ethyl-3-hydroxy-4(1H)-pyridinone **22**

M.p. 106–107 °C; IR (Nujol): 3 140 (OH), 1 738 (ester C=O), 1 620 (pyridinone C=O), 1 565 (C=C) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.04 [6H, d, -CH**$(CH_3)_2$**, *J* = 5.5Hz], 1.13 (3H, t, CH_2**CH_3**, *J* = 6.0Hz), 2.3–2.68 [1H, m, -**CH**$(CH_3)_2$], 2.75 (2H, q, **CH_2**CH_3, *J* = 6.0Hz), 4.27 (4H, br, s, N**CH_2CH_2**O), 5.55 (1H, br, s, **OH**), 6.15 (1H, d, **5–H**, *J* = 8.0Hz), 7.55 (1H. d, **6–H**, *J* = 8.0Hz); MS (EI): m/z, 253 [$M^{+\cdot}$]; Anal. Calcd. for $C_{13}H_{19}NO_4$: C, 61.65; H. 7.56; N, 5.53 Found C, 61.56; H, 7.45; N, 5.64%.

6.1.2.7. 1-[2´-(Pivaloyloxy)ethyl]-2-methyl]-3-hydroxy-4(1H)-pyridinone **25**

M.p. 139–140 °C; IR (Nujol): 3 150 (OH), 1 720 (ester C=O), 1 620 (pyridinone C=O), 1 570 (C=C) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.10 [9H, s, -C**$(CH_3)_3$**], 2.33 (3H, s, **2-CH_3**), 4.29 (4H, s, N**CH_2CH_2**O), 6.15 (1H, d, **5-H**, *J* = 7.5Hz), 6.35 (1H, br, s, **OH**), 7.56 (1H, d, **6-H**, *J* = 7.5Hz); MS (EI): m/z, 253 [$M^{+\cdot}$]; Anal. Calcd. for $C_{13}H_{19}NO_4$: C, 61.64; H, 7.56; N, 5.53 Found C, 61.74; H, 7.55; N, 5.41%.

6.1.2.8. 1-[2´-(Pivaloyloxy)ethyl]-2-methyl]-3-hydroxy-4(1H)-pyridinone hydrochloride **25** *HCl salt*

M.p. 186.5–188 °C; IR (Nujol): 3 140 (OH), 2 600 (OH), 1 725 (ester C=O), 1 630 (C=N) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.07 [9H, s, -C**$(CH_3)_3$**], 2.58 (3H, s, **2-CH_3**), 4.40 (2H, t, +NCH_2**CH_2**O), 4.69 (2H t, +N**CH_2**CH_2O), 7.45 (1H, d, **5-H**, *J* = 6.0Hz), 8.26 (1H, d, **6-H**, *J* = 6.0Hz), 9.8 (2H, br, **OH**).

6.1.2.9. 1-[3´-(Pivaloyloxy)propyl]-2-methyl-3-hydroxy-4(1H)-pyridinone **27**

M.p. 156–157 °C; IR (Nujol): 3 170 (OH), 1 720 (ester C=O), 1 620 (pyridinone C=O), 1 565 cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.10 [9H, s, -C**$(CH_3)_3$**], 1.80–2.15 (2H, m, CH_2**CH_2**CH_2O), 2.30 (3H, s, **2-CH_3**), 3.9–4.16 (4H, m, N**CH_2CH_2**O), 6.15 (1H, br, s, **OH**), 6.15 (1H, d, **5-H**, *J* = 7.5Hz), 7.55 (1H, d, **6-H**, *J* = 7.5Hz); MS (EI): m/z, 267 [$M^{+\cdot}$]; Anal. Calcd. for $C_{14}H_{21}NO_4$: C, 62.88; H, 7.92; N, 5.24 Found C, 62.89; H, 8.07; N, 5.20%.

6.1.2.10. 1-[3´-(Pivaloyloxy)propyl]-2-methyl-3-hydroxy-4(1H)-pyridinone hydrochloride **27** *HCl salt*

M.p. 152–153.4 °C; IR (Nujol): 3 100 (OH), 1 725 (ester C=O) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.13 [9H, s, -C**$(CH_3)_3$**], 1.90–2.30 (2H, m, +NCH_2**CH_2**CH_2O), 2.56 (3H, s, **2-CH_3**), 4.10 (1H, t, +NCH_2CH_2**CH_2**O), 4.45 (2H, t, +N**CH_2**CH_2CH_2O), 7.46 (1H, d, **5-H**, *J* = 6.0Hz), 8.33 (1H, d, **6-H**, *J* = 6.0Hz), 10.1 (2H, br, **OH**).

6.1.3. Synthesis of ester derivatives of 1-(hydroxyalkyl)-2-alkyl-3-benzyloxy-4(1H)-pyridinones. Method (b) (via carboxylic acid)

6.1.3.1. 1-[2´-(Benzoyloxy)ethyl]-2-ethyl-3-benzyloxy-4(1H)-pyridinone **18**

A solution of **9**, 1-[2´-(hydroxy)ethyl]-2-ethyl-3-benzyloxy-4(1*H*)-pyridinone, the free base (5.5 g, 20 mmol) and triphenylphosphine (5.8 g, 22 mmol, 1.1 eq) in tetrahydrofuran (200 mL) was added dropwise to a solution of diethyl azodicarboxylate (DEAD, 3.8 g, 22 mmol, 1.1 eq) and benzoic acid (2.6 g, 21 mmol, 1.05 eq) in distilled tetrahydrofuran (59 mL) at 0 °C with

stirring via a pressure equalising funnel under nitrogen for 45 min before the reaction was allowed to warm slowly to 20 °C and stirred for 18 h. Tetrahydrofuran was removed by rotary evaporation to give a yellow oil. The crude material was purified by column chromatography on silica gel (eluant, methanol:chloroform; 8:92, R_f = 0.55) to afford a pale yellow oil (7.6 g, > 95%). IR (nujol): 1 720 (ester C=O), 1 625 (pyridinone C=O), 1 570 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.03 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 2.65 (2H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 4.15 (2H, t, $N\mathbf{CH_2}CH_2O$), 4.49 (2H, t, $NCH_2\mathbf{CH_2}O$), 5.24 (2H, s, $\mathbf{CH_2}$Ph), 6.40 (1H, d, **5-H**, *J* = 8.0Hz), 7.20–7.65 (8H, m, **Ph** and benzoyl-***m,p*-H**), 7.32 (1H, d, **6-H**, *J* = 8.0Hz), 7.85–8.05 (2H, m, benzoyl-***o*-H**); MS (EI): m/z, 377 [$M^{+\cdot}$].

Analogous procedure reacting **9**, **10** and **11** with glacial acetic acid, pivalic acid and benzoic acid yielded the viscous oils of **16**–**20** respectively in a yield of > 95%.

6.1.3.2. 1-[2´-(Acetyloxy)ethyl]-2-ethyl-3-benzyloxy-4(1H)-pyridinone **16**

^{1}H-NMR ($CDCl_3$): δ 1.03 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 2.0 (3H, s, $\mathbf{CH_3}$), 2.62 (2H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 3.94–4.35 (4H, m, $N\mathbf{CH_2CH_2}O$), 5.27 (2H, s, $\mathbf{CH_2}$Ph), 6.36 (1H, d, **5-H**, *J* = 8.0Hz), 7.17–7.57 (6H, m, **ArH** & **6-H**); MS (EI): m/z, 315 [$M^{+\cdot}$].

6.1.3.3. 1-[3´-(Pivaloyloxy)ethyl]-2-ethyl-3-benzyloxy-4(1H)-pyridinone **17**

^{1}H-NMR ($CDCl_3$): δ 1.00 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 1.14 [9H, s, $-C\mathbf{(CH_3)_3}$], 2.63 (2H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 3.85–4.4 (4H, m, $N\mathbf{CH_2CH_2}O$), 5.24 (2H, s, $\mathbf{CH_2}$Ph), 6.37 (1H, d, **5-H**, *J* = 8.0Hz), 7.1–7.55 (6H, m, **ArH** & **6-H**).

6.1.3.4. 1-[(2´-Benzoyloxy)ethyl]-2-methyl-3-benzyloxy-4(1H)-pyridinone **19**

^{1}H-NMR ($CDCl_3$): δ 2.08 (3H, s, **2-**$\mathbf{CH_3}$), 4.10 (2H, t, $N\mathbf{CH_2}CH_2O$), 4.40 (2H, t, $NCH_2\mathbf{CH_2}O$), 5.18 (2H, s, $\mathbf{CH_2}$Ph), 6.45 (1H, d, **5-H**, *J* = 7.5Hz), 7.0–8.1 (11H, m, **ArH**, & **6-H**).

6.1.3.5. 1-[(3´-Benzoyloxy)propyl]-2-methyl-3-benzyloxy-4(1H)-pyridinone **20**

^{1}H-NMR ($CDCl_3$): δ 1.65–2.4 (2H, m, $NCH_2\mathbf{CH_2}CH_2O$), 2.10 (3H, s, **2-**$\mathbf{CH_3}$), 3.80 (2H, t, $N\mathbf{CH_2}CH_2CH_2O$), 4.20 (2H, t, $NCH_2CH_2\mathbf{CH_2}O$), 5.06 (2H, s, $\mathbf{CH_2}$Ph), 6.28 (1H, d, **5-H**, *J* = 7.5Hz), 6.9–8.1 (11H, m, **ArH** & **6-H**).

6.1.3.6. 1-[2´-(Benzoyloxy)ethyl]-2-ethyl-3-hydroxy-4(1H)-pyridinone **24**

The benzyl protected ester **18** (7 g, 18.6 mmol) was dissolved in dimethylformamide (150 mL) and Pd/C catalyst (5%, 0.7 g) was added. The solution was stirred at 20 °C under a constant stream of hydrogen for 24 h. The reaction mixture was then filtered and dimethylformamide was removed under high vacuum. The residue was treated with activated charcoal in chloroform, filtered and concentrated in vacuo to dryness. Recrystalisation from chloroform/diethylether afforded colourless crystals (4.9 g, 92%); m.p. 173–174 °C; IR (Nujol): 3 240 (OH), 1 720 (ester C=O), 1 620 (pyridinone C=O), 1 560 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.23 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 2.85 (2H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 4.25 (2H, t, $N\mathbf{CH_2}CH_2O$), 4.57 (2H, t, $NCH_2\mathbf{CH_2}O$), 6.40 (1H, d, **5-H**, *J* = 8.0Hz), 6.70 (1H, s, **OH**), 7.30 (1H, d, **6-H**, *J* = 8.0Hz), 7.4–7.65 (3H, m, benzoyl-***m,p*-H**), 7.85–8.08 (2H, m, benzoyl-***o*-H**); MS (EI):m/z, 287[$M^{+\cdot}$]; Anal. Calcd. for $C_{16}H_{17}NO_4$: C, 66.88; H, 5.96; N, 4.87 Found C, 66.78; H, 6.01; N, 4.92%.

6.1.3.7. 1-[(2´-Benzoyloxy)ethyl]-2-ethyl-3-hydroxy-4(1H)-pyridinone hydrochloride **24** *HCl salt*

M.p. 156–158 °C; IR (Nujol): 1 720, 1 635 cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.21 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 3.08 (1H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 4.55–4.95 (4H, m, $+N\mathbf{CH_2CH_2}O$), 7.50 (1H, d, **5-H**, *J* = 6.5Hz), 7.50–7.74 (3H, m, benzoyl-***m,p*-H**), 7.80–8.05 (2H, m, benzoyl-***o*-H**), 8.44 (1H, d, **6-H**, *J* = 6.5Hz), 9.25 (2H, br, **OH**).

Analogous hydrogenolysis reaction using **16, 17, 19** and **20** gave **21, 23, 26** and **28** respectively *(table III)*.

6.1.3.8. 1-[2´-(Acetyloxy) ethyl]-2-ethyl-3-hydroxy-4(1H)-pyridinone **21**

M.p. 126–127 °C; IR (Nujol): 3 200 (OH), 1 740 (ester C=O), 1 625 (pyridinone C=O), 1 585 (C=C) cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 1.23 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 2.04 (3H, s, $\mathbf{CH_3}$), 2.83 (2H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 4.02–4.47 (4H, m, $N\mathbf{CH_2CH_2}O$), 6.37 (1H, d, **5-H**, *J* = 8.0Hz), 6.77 (1H, br, s, **OH**), 7.28 (1H, d, **6-H**, *J* = 8.0Hz); MS (EI): m/z, 225 [$M^{+\cdot}$]; Anal. Calcd. for $C_{11}H_{15}NO_4$: C, 58.65; H, 6.71; N, 6.21 Found C, 58.49; H, 6.70; N, 6.21%.

6.1.3.9. 1-[2´-(Pivaloyloxy)ethyl]-2-ethyl-3-hydroxy-4(1H)-pyridinone hydrochloride **23** *HCl salt*

M.p. 184.5–186 °C; IR (Nujol): 3 120 (OH), 2 600 (OH), 1 720 (ester C=O), 1 630 (C=N) cm^{-1}; 1H NMR (DMSO-d_6): δ 1.07 [9H, s, $-C\mathbf{(CH_3)_3}$], 1.18 (3H, t, $CH_2\mathbf{CH_3}$, *J* = 6.0Hz), 3.01 (2H, q, $\mathbf{CH_2}CH_3$, *J* = 6.0Hz), 4.41 (2H, t, $+NCH_2\mathbf{CH_2}O$), 4.72 (2H, t, $+N\mathbf{CH_2}CH_2O$),

7.46 (1H, d, **5**–**H**, *J* = 6.5Hz), 8.26 (2H, d, **6**–**H**, *J* = 6.5Hz), 8.5 (2H, br, **OH**).

6.1.3.10. 1-[(2´-Benzoyloxy)ethyl]-2-methyl-3-hydroxy-4(1H)-pyridinone hydrochloride **26** *HCl salt*

M.p. 201–203 °C; IR (KBr): 3 502, 1 719, 1 630 cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 2.55 (3H, s, **2-CH_3**), 4.50 (4H, br, s, +N**CH_2CH_2**O), 7.10–7.95 (6H, m, **ArH** & **5-H**), 8.20 (1H, d, **6-H**, *J* = 6.0Hz), 7.9–9.6 (2H, br, **OH**); Anal. Calcd. for $C_{15}H_{16}NO_4Cl$: C, 58.16; H, 5.21; N, 4.52; Cl, 11.45 Found C, 58.38; H, 5.27; N, 4.45; Cl, 11.12%.

6.1.3.11. 1-[(3´-Benzoyloxy)propyl]-2-methyl-3-hydroxy-4(1H)-pyridinone hydrochloride **28** *HCl salt*

M.p. 168–170 °C; IR (KBr): 3 477, 1 710, 1 634 cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 2.0–2.5 (2H, m, NCH_2**CH_2**CH_2O), 2.55 (3H, s, **2-CH_3**), 4.0–4.7 (4H, m, +N**CH_2**CH_2**CH_2**O), 7.10–7.95 (6H, m, **ArH** & **5-H**), 8.15 (1H, d, **6-H**, *J* = 6.0Hz), 8.9–10.1 (2H, br, **OH**). Anal. Calcd. for $C_{16}H_{18}NO_4Cl$: C, 59.35; H, 5.60; N, 4.33; Cl, 10.95 Found C, 59.11; H, 5.64; N, 4.31; Cl, 10.68%.

6.2. Physicochemical and biological evaluation

6.2.1. Determination of distribution coefficients using the filter probe method. Free ligands

Distribution coefficients were determined using an automated continuous flow technique similar to that described by Tomlinson [24] by following the methodology as described by Rai and co-workers [14]. The system comprised an IBM compatible PC running the "TOPCAT" program [28, 29], which controlled both a Metrohm 665 Dosimat autoburette and a Pye-Unicam Lambda 5 UV/vis spectrophotometer, as well as performing all calculations of distribution coefficients. All distribution coefficient determinations were performed using AnalaR grade reagents under a nitrogen atmosphere using a flat-based glass vessel equipped with a sealable lid at 25 °C. The aqueous and octanol phases were presaturated with respect to each other before use. The filter probe consisted of a polytetrafluoroethylene (PTFE) plunger associated with a gel-filtration column. The aqueous phase (50 mM MOPS buffer, pH 7.4, prepared using Milli-Q water) was separated from the two phase system (1-octanol/MOPS buffer, pH 7.4) by means of a hydrophilic cellulose filter 5 μm diameter, 589/3 Blauband filter paper, Schleicher and Schuell) mounted in the gel-filtration column adjuster (SR 25/50, Pharmacia). A known volume (normally 25–50 mL) of MOPS buffer (saturated with octanol) is taken in the flat base mixing chamber. After a base line was obtained, the solution was used for reference absorbance. The compound to be examined was dissolved in buffer (saturated with octanol) so as to give an absorbance of between 0.5–1.5 absorbance units at the preselected wavelength (≈280 nm). The flow rate of the aqueous circuit was limited to 1 mL. The computer program calculates the partition coefficient ($D_{7.4}$) for each octanol addition.

6.2.2. Ester hydrolysis studies in phosphate buffer (pH 2 and 7.4), rat blood and liver homogenate

Each ester prodrug, 10 μmol in 0.1mL of acetonitrile solution (50% v/v in water), was added to 0.9 mL of phosphate buffer (PBS) (100 mM, pH 7.4 or pH 2.0). The samples were incubated at 37 °C for various time intervals up to 24 h. At the end of the incubation, the samples were removed from the incubator and immediately subjected to extraction as described below. Fresh heparinised rat whole blood was used throughout the present study. The whole blood (0.5 mL) was added in the phosphate buffer (0.5 mL, 0.139 M, pH 7.4) containing the ester substrate (2.0 μmol). Subsequently the samples were incubated at 37 °C for 0, 10, 20, 40 or 60 min. The hydrolytic reaction was terminated by removing the samples onto an ice bath and extracting the hydrolytic product immediately. The rat liver was removed from the animal and immersed in ice-cold PBS (100 mM, pH 7.4) in order to remove excess blood. The livers were blotted dry, weighed and cut into small pieces. Thereafter, the tissue was homogenised in PBS (100 mM, pH 7.4) in the ratio of 1 g liver tissue in 4 mL buffer. Similarly, each ester (2 μmol) was added to 1.0 mL of liver homogenate, which was diluted before use with PBS (100 mM, pH 7.4) to a concentration of 1 g liver tissue in 400 mL mixture. The incubation was carried out at 37 °C for 0, 10, 20, 40 or 60 min and the termination of the reaction was the same as for the blood samples.

6.2.2.1. Extraction and HPLC procedures for analysis of hydrolytic product

All of the incubated samples, in the PBS buffers, whole blood and liver homogenate, were added with organic solvent (acetonitrile:isopropanol = 80/20 v/v), solid NaCl and HCl solution (2 M, except the samples incubated in PBS with pH 2.0). The samples were then mixed and centrifuged. The upper organic layer was separated, evaporated and analysed by an established HPLC method. A Hewlett Packard Model 1090M Series-II HPLC system, complete with an auto injector, auto sampler and diode array detector, linked to a HP 900-300 data station was used in the present study. A polymer PLRP-S column (15 cm × 0.46 cm) and a gradient mobile phase system, utilising PBS (10 mM, pH 2.90, containing

2 mM EDTA) and acetonitrile, were used for the separation of the analytes. The eluents were monitored at 285 nm.

6.2.3. Method of iron mobilisation efficacy study in rat [25]

Hepatocytes of normal fasted rats (190–230 g) were labelled with ^{59}Fe by administration of ^{59}Fe-ferritin from tail vein. One hour later, each rat was administered orally with chelator (450 μmol/kg). Due to the poor water solubility associated with several hydrophobic prodrug molecules, 70% 1,2-propanediol in water (v/v) has been used as the solvent. The efficacy of **1** in the presence of 70% 1,2-propanediol has been compared. No detectable difference in iron excretion was induced in the presence of the solvent. Control rats were administered with an equivalent volume of 70% 1,2-propanediol. The rats were placed in individual metabolic cages and urine and faeces collected. Rats were allowed access to food 1 h after oral administration of chelator. There was no restriction of water throughout the study period. The investigation was terminated 24 h after the ^{59}Fe-ferritin administration, rats were sacrificed and the liver and gastrointestinal tract (including its content and faeces) were removed for gamma counting.

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